also do 102(b) on product.

Trypsin

Japanese Patent Office

102 & 103

(11)Publication number: 04282400
(43)Date of publication of application: 10/07/1992
(51)Int.Cl. C07K 15/06
A23L 1/305
A61K 37/64
C12N 9/99
C12P 21/06
(21)Application number: 03046431
(22) Date of filing: 03/12/1991
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(54) Angiotensin converting enzyme-inhibiting peptide

(57)Abstract:

Constitution: A peptide, which has angiotensin converting enzyme-inhibiting (ACE) activity, is separated from whey protein by Trypsin enzyme.
Effect: The peptide is safe for food and high in ACE activity. It is expected to treat or prevent hypertension by adding into medicines, or health and functional foods. etc.

(Range of patent petition)
(Petition 1): A peptide, which has angiotensin converting enzyme(ACE) -inhibiting activity, is separated from whey protein by Trypsin enzyme.

(Description of invention)
(0001)
(Area of the commercial use)
This invention is about new peptide has angiotensin converting enzyme(ACE) -inhibiting activity and treatment and prevention of hypertension.
(0002)
(Former technique) Many researchers have recognized that renin-angiotensin system is significant to hypertension and circulatory disorder. Renin-angiotensin has ACE. It forms angiotensinII, which contracts blood vessels, from the carboxyl terminal of oligopeptide•angiotensinI. ACE also breaks up hypotension peptide and bradykinin to increase blood pressure. Therefore, stopping forming of angiotensinII, in another word, deactivating activity of ACE, is suppressing the hypertension. As mentioned above, researching and developing on natural and synthetic ACE inhibitor, for example, captopril, has been progressing.
(0003)Because ACE is an aminopeptidase to separate dipeptide, the peptide, which has affinity of bond of ACE, is the inhibitor of ACE. Proteins used to be only nutrient absorbed by intestine as amino acid, but today some of the proteins absorbed as oligopeptide are affect on hormone in the body. As results, many researches have been researching on food-derived bioactive peptide. Public knows today that ACE inhibitor peptide is in hydrolyzed casein and soybean, but it is not known about the result of the effect of doses for oral administration.
(0004)
(Object of invention) To invent the peptide which treat and prevent hypertension. even by oral administration.
(0005)
(Methods)Providing the peptide, from tripsin-hydrolized whey protein , has ACE inhibitor activity.
(0006)Details
(0007)This ACE inhibitor peptide is made from trypsin-hydrolized whey protein.

(0008)The molecular weight of ACE inhibitor peptide from trypsin-hydrolized whey protein is less than about 1000. Starting ingredient is cheese or acid whey. Trypsin is one of the proteinase for general use. The concentration of the trypsin to whey protein is from 0.1~1% by weight.
(0009)Procedure of the peptide
(0010)After taking out small molecular material, react whey protein and trypsin at pH6 ~9 at 25~50C for 8 ~24hours, and taking out trypsin and non-hydrolyzed protein by using heat and acid treatment. Add methanol and ethanol to extract the peptide. It is better to use ultra filtration membrane to take out large peptide after getting rid of organic materials. Use reverse resin column, Cosmosil C18-opn, to adsorb the peptide and desorb it by using methanol to purify it. Other methods are using high-pressure liquid, gel osmosis, or ion exchange chromatography.
(0011) The peptide can be liquid or powder. It can be mixed with hydrochloric acid, sulfuric acid, succinic acid, citric acid, and tartaric acid as food grade salts.
(0012)This peptide can be used for oral administration as healthy and functional food, and for intravenous injection. The amount of recommended dose for human per day is more than 100mg of solid of the peptide. This peptide is safe to take everyday because it is from natural ingredient.
(0013)
(Effect) The peptide is safe for food and high in ACE activity. It is expected to treat or prevent hypertension by adding into medicines, or health and functional foods, etc.
(0014)
(Example1)After dissolving 100g of cheese whey powder into 1L of distilled water, removing small molecules, riboflavin and lactose, by using dialysis tube (molecular cut-off 12000~14000) in running water at 4C for 2 days. Next, adjust pH of retentate to 8.0 with 1N NaOH, and add CaCl to adjust final concentration to 5mM. Heat to 37C, add 100mg of trypsin(Sigma type XIII from Bovine Pancreas, TPCK, 12200 BAEE unit/mg protein), and stir for 12 hours to hydrolyze. And then, add hydrochloric acid to adjust pH 3.0 to precipitate non-hydrolyzed protein and trypsin, and centrifuge it at 8000rpm for 30minutes to take out precipitated materials. Next, use ultra filtration membrane (Advantech Toyo, Inc. UHP-150, membrane: Fuji Filter Industry, Inc. molecular cut-off 10000) to remove large molecules. Then, use reverse resin column(Cosmos IL C18-OPN by Nakaraitesk, Inc. Ø3.5X50cm) with buffer of 0.01weight%TFA to adsorb ACE inhibitor peptide, and desorb it by using 0.01weight%TFA-methanol 0.2%/min. liner gradient method at 3ml per min. of flow. Collect the eluate from 10min. to 50 min. of gradient time that has most ACE inhibitor protein, remove methanol by vacuum concentration, and freeze dry the eluate to make 100mg of white powder.
(0015)Analyzing the peptide by using HPLC
The result is on the figure 1.
(0016)Analyzing conditions
Column:R-ODS-5 4.6X250mm by YMC, Inc.
A buffer: 0.01%TFA solution
B buffer: acetonitrile
Flow: 1ml/min.
(0017)
(Test 1)Transfer 0.04ml of the ACE inhibitor peptide from example 1into a test tube, and add 0.1M boric acid buffer (including 0.3M NaCl, pH8.3). Next, add 0.2ml of hippuryl-His-Leu (Sigma) to adjust final concentration to 5mM, and heat at 37C for 10min. And then, add 0.04ml of 25mU/ml of ACE from rabbit lung (Sigma), and react it at 37C for 30min. After that, add 0.25ml of 1N hydrochloric acid to stop the reaction. And then, add 1.7ml of ethyl acetate, stir it for 30 sec, and centrifuge it at 3000rpm for 10min.to collect 1.4ml of the layer of ethyl acetate. After removing the solvent of it by heating at 120C for 40min. add 1ml of distilled water to extract hyppuryl acid. Finally, measure the absorption of the acid at 228nm, and use this as enzyme activator.
(0018)Measure enzyme activity of the peptide from example1(the concentrate unit, μg/ml, of 50% ACE inhibiting activity) by using the formula below
ACE inhibiting activity = (A-B)/(A-C) X100%
A: Enzyme activity with no peptide (228nm)
B: Enzyme activity with the peptide (228nm)
C: Activity without enzyme and the peptide (228nm)
Result: ACE inhibiting activity = 40μg/ml

(0019)
(Test 2)Measuring the blood pressure of 5 of 12 weeks old, male, natural hypertension rats (SHR) (Nihon Charles river, Inc. 1 group of 5). They have been fed with food and water freely.
Measure blood pressure 6 hours after forced feeding 125mg, 250mg, and 500mg per 1kg weight of the peptide from example 1 solution (mixed with physiological saline) with a tube.
Use non-visible blood pressure instrument(PE-300 by NARCO BIO-SYSTEM, Inc.), and measure highest blood pressure by tail-cuff method.
(0020)
(Table 1)

Amount of the peptide solution (mg/kg)	Highest blood pressure ±SE (mmHg)
0	222.3±2.0
125	218.1±7.0
250	216.5±3.7
500	201.6±3.5 *

*There is 0.1% of critical rate.

(0021)
(Test 3)Use 3 of 12 weeks old, male, SHR have not been fed for a day, and feed forcedly 1000mg /kg weight of the peptide solution from example 1 with a tube. 6 of Control SHR are fed only physiological saline.
(0022)
(Table 2)

	Highest blood pressure±SE (mmHg)	
	Control SHR	The peptide fed SHR
Before feeding	211.8±3.5	
After 2 hours	214.4±2.9	198.0±7.2
After 3 hours	212.1±2.3	195.8±8.0
After 5 hours	212.9±3.6	187.8±6.8
After 7 hours	211.2±3.6	181.1±2.7
After 24 hours	217.0±3.7	218.0±3.5

(0023)The results of test 2 and 3 indicate the peptide from example 1 can lower the blood pressure of the peptide fed SHR.
(0024)
(Test 4)Use 6 of 12 weeks old, male, SHR after same condition as test 3, fill 21.4mg/ml of the peptide from example 1 with distilled water in feeding bottle, and feed freely for 6 days.
The average consumption is about 360mg (same as 1000mg/1kg weight). Feeding food is no restriction. After 6 days, the SHR are fed with water. Measuring blood pressure is once a day, and the average is on table 3
(0025)
(Table 3)

Highest blood pressure±SE (mmHg)			
	The peptide fed SHR	Control SHR	Differences
0 day	232.6±0.6		
1st day	229.0±6.8	223.2±3.1	5.8
4th day	209.4±2.5	224.9±2.8	-15.5*
5th day	209.6±4.2	228.1±2.0	-18.5*
6th day	206.1±2.5	223.2±3.1	-17.1*
1st day after finishing feed	215.0±2.5	220.3±5.2	-5.3
2nd day after finishing feed	214.9±8.2	213.9±1.5	1

*There is 1% of critical rate.

(0026)The table 3 shows that feeding small amount of the peptide as well gradually decreases the blood pressure. After stopping feeding it, blood pressure is increased little by little until the same blood pressure level before the test.

(figure 1)
The HPLC result of ACE inhibitor peptide from example 1.